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Polymorphisms of *STAT5A* gene and their association with milk production traits in Holstein cows

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Abstract The *STAT5A* gene was studied as a candidate gene for five milk production traits (milk yield at 305 days, protein percentage, fat percentage, lactose percentage and dry matter percentage) in Holstein cows. According to the sequence of bovine *STAT5A* gene, two pairs of primers (P1 and P2) were designed to detect polymorphisms of *STAT5A* gene in 401 Holstein cows by PCR-RFLP and PCR-SSCP. The results showed that the products amplified by primers P1 and P2 displayed polymorphisms. For P1, three genotypes (AA, AG, and GG) were detected, and the frequency of AA/AG/GG was 0.252/0.486/0.262, respectively. Sequence analysis revealed a single nucleotide substitution A–G at 14217 bp (GenBank NC_007317) of bovine *STAT5A* gene while compared GG genotype with AA genotype. The differences of the least squares means for the four milk production traits (milk yield at 305 days, fat percentage, lactose percentage and dry matter percentage) between AA, AG and GG were not significant ($P > 0.05$). Least squares mean of protein percentage for AG or GG was significantly higher than that for AA ($P < 0.05$); the difference of the least squares mean for protein percentage was not significant between AG and GG ($P > 0.05$). For P2, three genotypes (CC, CT, and TT) were detected in Holstein cows, and the frequency of CC/CT/TT was 0.751/0.234/0.015, respectively. Sequencing revealed an insertion CCT at 17266 (NC_007317) of bovine *STAT5A* gene while compared CC genotype with TT genotype. The differences of the least squares means for the three milk production traits (protein percentage, lactose percentage and dry matter percentage) between CC, CT and TT were not significant ($P > 0.05$). Least squares mean of milk yield at 305 days for TT or CT was significantly higher than that for CC ($P < 0.05$); the difference of the least squares mean for milk yield at 305 days was not significant between TT and CT ($P > 0.05$). Least squares mean of fat percentage for CC or CT was significantly higher than that for TT ($P < 0.05$); the difference of the least squares mean for fat percentage was not significant between CC and CT ($P > 0.05$). The results preliminarily indicated that allele G of A14217G polymorphic site of *STAT5A* gene is a potential DNA marker for improving protein percentage in dairy cattle, 17266indelCCT polymorphic site of *STAT5A* gene is a potential DNA marker for improving milk yield at 305 days and fat percentage in dairy cattle.

Keywords Holstein cows · *STAT5A* gene · Polymorphism · Milk production traits

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Introduction

The members of signal transducer and activator of transcription (STAT) family are involved in cell proliferation,

differentiation and apoptosis. STAT5 contains two closely related subtypes, STAT5A and STAT5B, which show 96% similarity [1]. The two subtypes can form heterodimers after phosphorylation while they are encoded by two separate genes [2, 3]. The deletion of *STAT5A* makes mammary dysplasia and diminished lactation capacity [4], while *STAT5B* deletion does not affect the development of the mammary gland [5]. Bole-Feysot et al. [6] found that STAT5 dimer could combine with the γ-interferon activation sites (GAS) located in the promoter region of the milk protein gene and activate transcription. Therefore they considered that *STAT5* gene may be associated with milk protein yield and milk protein percentage. In addition, *STAT5* gene has a wide range of physiological functions as an important transcription factor. A variety of cytokines such as interleukin-2, interleukin-3, interleukin-12, erythropoietin, granulocyte colony-stimulating factor, granulocyte–macrophage colony stimulating factor, thrombopoietin and prolactin and growth hormone can activate STAT5 [7]. The study found that STAT5 involved in the regulation of hematopoietic system, immune regulation, but also promoting breast development and growth and development [8–12]. Bovine *STAT5A* gene is located on chromosome 19 and contains 19 exons, encoding 794 amino acids. The expression of *lactoprotein* gene in bovine mammary epithelial cell is mainly mediated by the transcription factor STAT5A [13–15]. *STAT5A* gene is a potential quantitative trait locus for milk production traits.

The objectives of the present study were to identify polymorphisms of *STAT5A* gene and to analyze association between the polymorphisms of the *STAT5A* gene and milk production traits in Chinese Holstein cows. The results could provide evidence for marker-assisted selection in cow breeding programs.

Materials and methods

Blood sample collection and DNA preparation

Blood samples (10 ml per cow) were collected from 401 Chinese Holstein cows, which were randomly selected from four cow herds in Hebei province, P.R. China. Genomic DNA was extracted from whole blood by the phenol–chloroform method, and then dissolved in TE buffer (10 mmol/l Tris–HCl (pH 8.0), 1 mmol/l EDTA (pH 8.0)) and kept at −20°C.

Primer sequences and polymerase chain reaction (PCR) amplification

Two pairs of primers, P1 and P2, were designed according to He et al. [16]. These primers were synthesized by Shanghai Invitrogen Biotechnology Limited Corporation (Shanghai, China). Primer sequence, PCR product size and amplified region were listed in Table 1.

PCR was carried out in 25 μl volume containing approximately 2.5 μl of 10× PCR buffer (50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.0), 0.1% Triton X-100), 1.5 mmol/l $MgCl_2$, 200 μmol/l each dNTP, 1 μmol/l each primer, 50 ng genomic DNA, and 1 U *Taq* DNA polymerase (SABC, Beijing, China). PCR conditions were as follows: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s; with a final extension at 72°C for 10 min on Mastercycler® 5333 (Eppendorf AG, Hamburg, Germany).

Restriction fragment length polymorphism (RFLP) analysis

Restriction enzyme reaction was carried out in 15 μl volume. The PCR products (7 μl) were digested with 0.5 μl *Msp*A1I (New England Biolabs, Beverly, MA, USA) for 5 h at 37°C. The digested products were separated by electrophoresis in 3% agarose gel (Promega) in parallel with a 600 bp DNA marker.

Single-strand conformational polymorphism (SSCP) detection

A volume of 3 μl PCR product was transferred into an Eppendorf tube, mixed with 7 μl gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mmol/l EDTA (pH 8.0), 10%

Table 1 Primer sequence, product size, product position and annealing temperature of PCR amplification for bovine *STAT5A* gene

Primer	Primer sequence (5′–3′)	Product size (bp)	Product position[a]	Annealing temperature (°C)
P1	F: ccagggtgcatacaggacag	224	Intron 9–Intron 10	58
	R: gcaggttacgaggactcagg			
P2	F: cttgggagaacctaacatcact	379	Intron 15–Exon 16	58
	R: agacctcatccttgggcc			

[a] Base positions corresponding to NC_007317 of GenBank

glycerol. The mixture was agitated and denatured at 98°C for 10 min, and quickly chilled on ice block for 7 min and loaded onto 12% neutral polyacrylamide gels (acrylamide:bisacrylamide = 39:1). Electrophoresis was performed in 1× Tris borate (pH 8.3)–EDTA buffer at 110 V for 17–22 h at room temperature. After electrophoresis, the DNA fragments in the gels were visualized by silver nitrate staining, photographed and analyzed using an AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA). SSCP genotypes were identified by mobility shift due to conformational difference of the single-stranded DNAs of the amplified fragments by each primer, which is caused by nucleotide variation.

Cloning and sequencing

After PCR products of P1 and P2 were analyzed by RFLP and SSCP respectively, PCR products of different homozygous genotypes were separated on 0.8% agarose gels and recovered using Gene clean II kit (Promega, Madison, WI, USA). Each DNA fragment was ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions at 4°C overnight. The ligation reactions were performed in 10 μl volume containing PCR product 1 μl, pGEM-T Easy vector (50 ng/μl) 1 μl, T_4 ligase (3 U/μl) 1 μl, 2× ligation buffer 5 μl, ddH_2O 2 μl. The recombinant DNA was then transformed into *Escherichia coli* DH5α competent cell. Positive clones of transformed cells were identified by PCR amplification. Five clones of each homozygous genotype were selected and sequenced. Each clone was sequenced for three times. The target DNA fragments in recombinant plasmids were sequenced from both directions using an automatic ABI 377 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) by GENEWIZ, Inc. Beijing.

Statistical analysis

The following statistical model was fitted to compare difference of five milk production traits (milk yield at 305 days, protein percentage, fat percentage, lactose percentage and dry matter percentage) among *STAT5A* genotypes in Holstein cows.

$$y_{ijklm} = \mu + S_i + H_j + P_k + G_l + e_{ijklm}$$

where y_{ijklm} is phenotypic value of milk production trait, μ is population mean, S_i is the fixed effect of the ith bull, H_j is the fixed effect of the jth dairy farm ($j = 1, 2, 3,$ and 4), P_k is the fixed effect of the kth parity ($k = 1, 2,$ and 3), G_l is the fixed effect of the lth genotype, e_{ijklm} is the random error effect of each observation. Calculations were achieved using Proc GLM (General Linear Model) of SAS (V 8.12).

Results

PCR amplification of bovine *STAT5A* gene

Genomic DNA of Holstein cow was successfully amplified using two pairs of primers for *STAT5A* gene. The PCR products were separated on 1.5% agarose gels. The results showed that amplification fragment sizes were consistent with the target ones and had a good specificity, which could be directly analyzed by RFLP or SSCP. PCR products amplified by the two pairs of primers were shown in Fig. 1.

*Msp*A1I analysis of bovine *STAT5A* gene

The uniform fragment of 224 bp by the amplification of P1 was obtained after 2% agarose gel electrophoresis in Holstein cows. The 224 bp PCR products were completely digested with the restriction endonuclease *Msp*A1I and genetic polymorphisms (G14217A) were investigated by PCR-RFLP. According to sequence analysis and restriction endonuclease map (Fig. 2), three genotypes were detected in 401 Holstein cows, AA (37/41/146 bp), AG (37/41/78/146 bp) and GG (78/146 bp).

SSCP analysis

Analysis by SSCP indicated that the PCR products amplified by primer P2 displayed polymorphisms. Three genotypes (CC, CT, and TT) were detected by primer P2 (Fig. 3).

Sequencing of different genotypes and nucleotide mutations

For primer P1, sequencing revealed a single nucleotide substitution A into G at 14217 bp (GenBank NC_007317) of bovine *STAT5A* gene while compared GG genotype with AA genotype (Fig. 4). For primer P2, Sequencing revealed

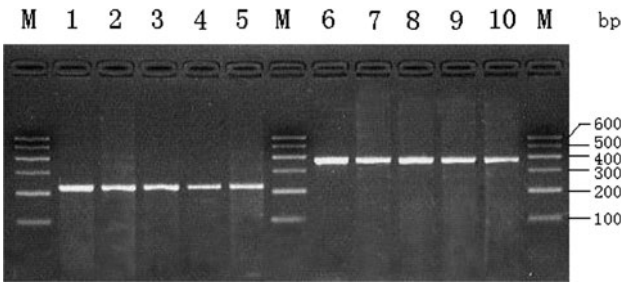

Fig. 1 PCR amplification products of primers P1 and P2. *Lanes 1–5* PCR products of P1; *Lanes 6–10* PCR products of P2; *M* 600 bp DNA marker

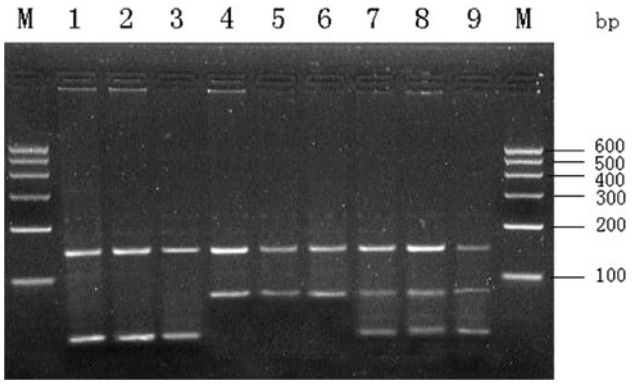

Fig. 2 *Msp*A1I-RFLP patterns of PCR products of P1 site in 3% agarose gel. *Lanes 1–3* AA genotype; *Lanes 4–6* GG genotype; *Lanes 7–9* AG genotype; *M* 600 bp DNA marker

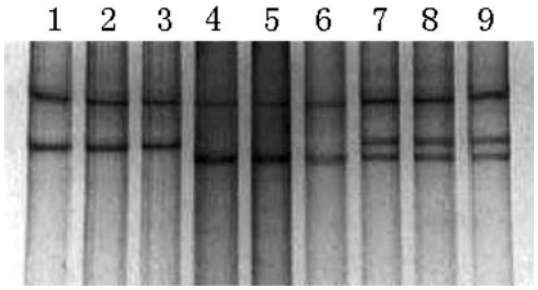

Fig. 3 SSCP analysis of PCR amplification products of primer P2 in bovine *STAT5A* gene. *Lanes 1–3* CC genotype; *Lanes 4–6* TT genotype; *Lanes 7–9* CT genotype

an insertion mutation (17266indelCCT) between genotype CC and genotype TT (Fig. 5).

Allele and genotype frequencies of *STAT5A* gene

Allele and genotype frequencies of *STAT5A* gene in 401 Holstein cows were presented in Table 2.

Genetic characteristics of *STAT5A* gene

Genetic characteristics of *STAT5A* gene in 401 Holstein cows were presented in Table 3.

The genotype distributions of the two polymorphisms analyzed were in Hardy–Weinberg equilibrium in Holstein cows (data shown in Table 3). The genotype frequencies of the two polymorphic sites were not affected by selection, mutation or migration and other factors.

Association of the *STAT5A* gene polymorphisms with milk production traits

The least squares means and standard errors for the five milk production traits (milk yield at 305 days, protein percentage, fat percentage, lactose percentage and dry matter percentage) of different genotypes of *STAT5A* gene in Holstein cows were given in Table 4.

Table 4 shows that, for P1, the differences of the least squares means for the four milk production traits (milk yield at 305 days, fat percentage, lactose percentage and dry matter percentage) between AA, AG and GG were not significant ($P > 0.05$). Least squares mean of protein percentage for AG or GG was significantly higher than that for AA ($P < 0.05$); the difference of the least squares mean for protein percentage was not significant between AG and GG ($P > 0.05$). For P2, the differences of the least squares means for the three milk production traits (protein percentage, lactose percentage and dry matter percentage) between CC, CT and TT were not significant ($P > 0.05$). Least squares mean of milk yield at 305 days for TT or CT was significantly higher than that for CC ($P < 0.05$), the difference of the least squares mean for milk yield at 305 days was not significant between TT and CT ($P > 0.05$). Least squares mean of fat percentage for CC or CT was significantly higher than that for TT ($P < 0.05$);

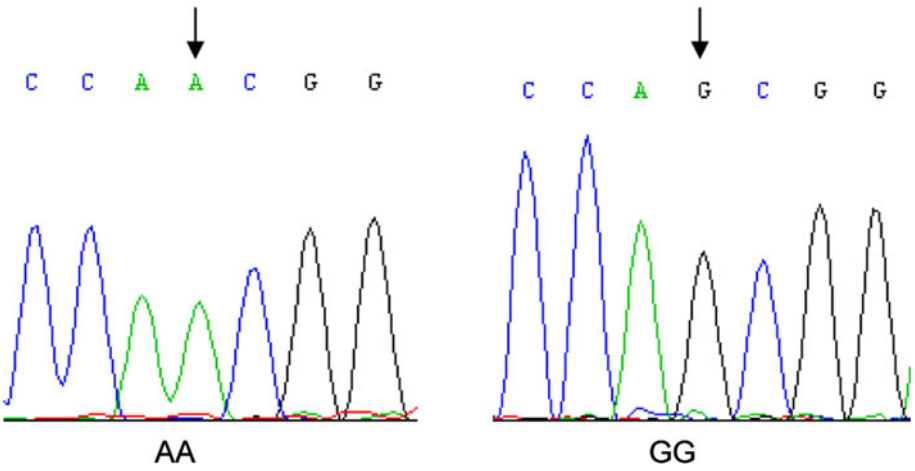

Fig. 4 Sequence comparison of AA and GG genotypes of primer P1 in bovine *STAT5A* gene

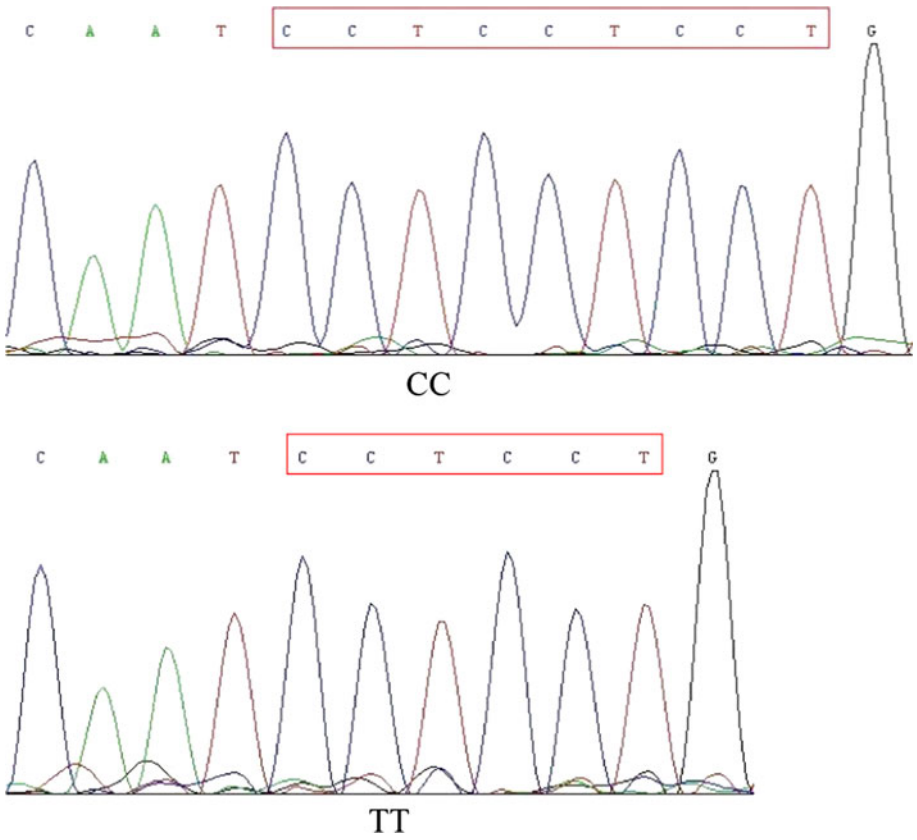

Fig. 5 Sequence comparison of CC and TT genotypes of primer P2 in bovine *STAT5A* gene

Table 2 Allele and genotype frequencies of two SNPs of *STAT5A* gene in Holstein cows

Primer	Genotype	Number of samples	Genotype frequency	Allele	Allele frequency
P1	AA	101	0.252	A	0.495
	AG	195	0.486		
	GG	105	0.262	G	0.505
P2	CC	301	0.751	C	0.868
	CT	94	0.234		
	TT	6	0.015	T	0.132

Table 3 Genetic characteristics of two polymorphic sites in Holstein cows

Primer	χ^2 (*P*)	Polymorphism information content	Heterozygosity	Effective number of alleles
P1	0.30 (0.861)	0.375	0.500	2.000
P2	0.19 (0.909)	0.203	0.229	1.298

the difference of the least squares mean for fat percentage was not significant between CC and CT ($P > 0.05$).

Discussion

Polymorphism of bovine *STAT5A* gene

Antoniou et al. [17] reported the polymorphisms of the bovine *STAT5A* gene. Flisikowski et al. [18] found two mutations, 12550indelCCT and T12743C. The SNP T12743C leaded to the restriction endonuclease site appeared of *Msl* I, and resulted in an amino acid change of valine → alanine as well. Brym et al. [15] reported a nucleotide mutation (A9501G) in intron 9 of bovine *STAT5A* gene. He et al. [16] found that there were two linked single nucleotide mutations on the bovine STAT5A genomic sequence (AJ237937) in Holstein cows, the substitution T/C at position 12440 and the insertion CCT at 12550. Khatib et al. [19] identified twelve mutations in the

Table 4 Least squares means and standard errors for milk production traits of different genotypes of *STAT5A* gene in Holstein cows

Primer	Genotype	Number of samples	Milk yield at 305 days (kg)	Protein percentage (%)	Fat percentage (%)	Lactose percentage (%)	Dry matter percentage (%)
P1	AA	101	$7229.4^{a} \pm 247.2$	$2.92^{b} \pm 0.10$	$3.68^{a} \pm 0.12$	$4.71^{a} \pm 0.07$	$12.22^{a} \pm 0.21$
	AG	195	$7052.6^{a} \pm 204.7$	$3.15^{a} \pm 0.06$	$3.70^{a} \pm 0.07$	$4.72^{a} \pm 0.04$	$12.23^{a} \pm 0.15$
	GG	105	$6949.2^{a} \pm 228.7$	$3.18^{a} \pm 0.08$	$3.73^{a} \pm 0.09$	$4.75^{a} \pm 0.05$	$12.25^{a} \pm 0.18$
P2	CC	301	$6905.5^{b} \pm 130.4$	$3.11^{a} \pm 0.03$	$3.72^{a} \pm 0.05$	$4.74^{a} \pm 0.04$	$12.24^{a} \pm 0.12$
	CT	94	$7500.1^{a} \pm 210.2$	$3.07^{a} \pm 0.09$	$3.69^{a} \pm 0.09$	$4.71^{a} \pm 0.06$	$12.20^{a} \pm 0.19$
	TT	6	$8750.2^{a} \pm 280.2$	$3.01^{a} \pm 0.15$	$2.97^{b} \pm 0.12$	$4.67^{a} \pm 0.09$	$12.15^{a} \pm 0.24$

Least squares means with the same superscript letters for the same site have no significant difference ($P > 0.05$). Least squares means with the different superscripts letters for the same site differ significantly ($P < 0.05$)

bovine STAT5A gene. Bao et al. [20] detected a C/T substitution located at position 12735 on the bovine STAT5A genomic sequence(AJ237937, which determine amino acid substitution of threonine to isoleucine. Selvaggi et al. [21] reported a genetic polymorphism of *STAT5A* protein, a substitution C > T at position 6853 within exon 7 in Italian Brown cattle.

In the present study, two mutations, G14217A and 17266indelCCT, were identified. The mutation G14217A was accorded with the A9501G reported by Brym et al. [15]. In addition, the mutation 17266indelCCT was accorded with the 12550indelCCT reported by Flisikowski et al. [18] and He et al. [16]. But the mutation T12440C in intron 9 linked with the 12550indelCCT reported by He et al. [16] was not found in this study. It may be due to the variety of cattle breeds.

Relationship of *STAT5A* gene with economic trait of Holstein cows

Liu et al. [4] identified that *STAT5A* knock-out mice exhibit defective mammary gland development and milk secretion disorder, STAT5A were involved in adult mammary gland development and lactogenesis of mice. He et al. [16] reported that the mutation A9501G of bovine *STAT5A* gene was associated with milk yield, milk protein yield and fat percentage ($P < 0.10$, $P < 0.05$) in Chinese Holstein cows, while Brym et al. [15] noted that it was not significantly associated with any of the milk performance traits (milk yield, fat yield, fat content, protein yield and protein content) ($P > 0.05$) in Black-and-White cows, but has significant associations between milk yield, fat and protein content in the first and second lactations of Jersey cows. In addition, Bao et al. [20] found that the mutation A9501G had strong effects on milk yield at 305 days and protein percentage, but had no significant associations between fat percentages. The two linked mutations, T12440C and 12550indelCCT, were significantly associated with milk yield, fat yield and protein yield [16]. The SNP C12195G in exon 8 of bovine *STAT5A* gene showed significant associations with milk protein and fat percentage, however, the SNP A14217G in intron 9 was not significantly associated with milk production traits (fat yield, fat percentage, milk yield, protein yield, protein percentage and somatic cell score (SCS)) [19]. Mao et al. [22] found that STAT5 were involved in acting prolactin, thus the single nucleotide polymorphisms of *STAT5* might affect the milk composition by coordinating fatty acid and protein content. Therefore, *STAT5* genes could be potential genetic markers for milk production traits. Selvaggi et al. [21] reported a genetic polymorphism of *STAT5A* protein, a substitution C > T at position 6,853 within exon 7 in Italian Brown cattle, CC cows produced more milk than CT and protein content was higher while CC compared with CT genotypes.

The study performed an association analysis between *STAT5A* gene variants and milk yield at 305 days, protein percentage, fat percentage, lactose percentage and dry matter percentage. The results showed that allele G of A14217G on *STAT5A* gene was significantly associated with increased protein percentage in Holstein cows ($P < 0.05$). This finding was in agreement with Bao et al. reported [20], however, it was quite different with references [15, 16, 19]. This was probably related to the group difference or various external factors such as dairy cattle feeding and management level. In addition, the study still showed that 17266indelCCT polymorphic site had strong effect on milk yield at 305 days and fat percentage ($P < 0.05$). It was accorded with the result of He et al. [16]. In conclusion, it was suggested A14217G and 17266indelCCT mutations of *STAT5A* gene as potential DNA markers and *STAT5A* as a candidate gene that could be used in selection programs for milk production traits in dairy cattle.

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